Distinct Functional and Anatomical Architecture of the Endocannabinoid System in the Auditory Brainstem

Yanjun Zhao¹, Maria E. Rubio³, Thanos Tzounopoulos¹,²

¹Department of Otolaryngology, ²Center for the Neural Basis of Cognition, University of Pittsburgh and Carnegie Mellon, Pittsburgh, Pennsylvania 15261, ³Department of Physiology and Neurobiology, University of Connecticut, Storrs, Connecticut 06269

Correspondence and requests for materials should be addressed to TT: thanos@pitt.edu

Running Title: Endocannabinoid Signaling in the Auditory System

Keywords: dorsal cochlear nucleus, endocannabinoids, CB1, synaptic plasticity, feedforward inhibition

Acknowledgments: We thank Dr. John Keen for helping to initiate this project, Drs. Karl Kandler, Kuei Tseng and Jake Hanchar for critical reading of the manuscript, and Dr. Ken Mackie for providing antibodies.

Grants: This work was supported by NIH/NIDCD grants R01 DC007905 to TT and DC006881 to MER. NSF DBI-0420580 contributed funds to purchase the Tecnai 12 Biotwin.
**Abstract.** Endocannabinoids (ECs) act as retrograde messengers that enable postsynaptic cells to regulate the strength of their synaptic inputs. Here, by using physiological and histological techniques, we revealed that, unlike in other parts of the brain, excitatory inputs are more sensitive than inhibitory inputs to EC signaling in the dorsal cochlear nucleus (DCN), an auditory brainstem nucleus.

The principal cells of the DCN, fusiform cells, integrate acoustic signals through non-plastic synapses located in the deep layer with multimodal sensory signals carried by plastic parallel fibers in the molecular layer. Parallel fibers contact fusiform cells and inhibitory interneurons, the cartwheel cells, which in turn inhibit fusiform cells. Postsynaptic depolarization or pairing of postsynaptic potentials (PSPs) with action potentials (APs) induced EC-mediated modulation of excitatory inputs, but did not affect inhibitory inputs. Quantitative electron microscopical studies revealed that glutamatergic terminals express more cannabinoid 1 receptors (CB1Rs) than glycinergic terminals. Fusiform and cartwheel cells express diacylglycerol lipase α and β (DGLα/β), the two enzymes involved in the generation of the EC, 2-arachidonoyl-glycerol (2-AG). DGLα and DGLβ are found in the spines of cartwheel but not fusiform cells indicating that the synthesis of ECs is more distant from parallel fiber synapses in fusiform than cartwheel cells. The differential localization and density of DGLα/β and CB1Rs leads to cell- and input-specific EC signaling that favors activity-dependent EC-mediated suppression at synapses between parallel fibers and cartwheel cell spines, thus leading to reduced feedforward inhibition in fusiform cells. We propose that EC signaling is a major modulator of the balance of excitation and inhibition in auditory circuits.
Introduction

Retrograde endocannabinoid signaling is one of the major activity-dependent neuromodulatory systems in the brain (Chevaleyre et al., 2006; Hashimotodani et al., 2007). Endocannabinoids (ECs) are released from postsynaptic neurons and modulate synaptic transmission of excitatory and inhibitory inputs. This modulation can be short- or long-term and is mediated by presynaptic, G protein-coupled, cannabinoid receptors (CB1Rs) (Freund et al., 2003; Piomelli, 2003; Chevaleyre et al., 2006). In many cases, ECs are released after postsynaptic depolarization, which leads to depolarization-induced suppression of inhibitory and excitatory inputs (DSI and DSE, respectively; Llano et al., 1991; Pitler and Alger, 1993; Wilson and Nicoll, 2001; Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001). In addition, synaptic activation with brief bursts can evoke endocannabinoid release and lead to synaptically evoked suppression of excitation (SSE) (Brown et al., 2003; Melis et al., 2004; Brenowitz and Regehr, 2005; Margaggi and Attwell, 2005).

Understanding the physiological role of EC signaling requires the study of the anatomical and functional properties of EC system at excitatory and inhibitory inputs. Structure-function studies of EC systems in the hippocampus, cerebellum and striatum have revealed that EC signaling is more prominent at inhibitory terminals as a result of increased presynaptic CB1R expression (Ohno-Shosaku et al., 2002; Brenowitz et al., 2006; Yoshida et al., 2006; Uchigashima et al., 2007).

Behavioral studies have revealed that cannabis users show deficits in their ability to detect target tones of particular location, pitch and duration from a total sample of random frequencies (Kempel et al., 2003). In addition, impairment in auditory sensory
gating response has been observed in rats treated with CB1R agonists (Hajos et al., 2008). Despite the known effects of cannabinoids in regulating acoustic discrimination and auditory perception, little is known about the organization and function of EC signaling on auditory circuits. The dorsal cochlear nucleus (DCN), an auditory brainstem nucleus resembling the cerebellum (Oertel and Young, 2004), is well suited to testing the anatomical and functional properties of EC signaling on auditory circuits. The DCN represents a site where cell-specific EC signaling determines cell-specific short- and long-term synaptic plasticity (Tzounopoulos et al., 2007). The DCN integrates auditory with non-auditory input, and is thought to play a role in the orientation of the head towards sounds of interest and in the suppression of self-generated sounds (Sutherland et al., 1998; May, 2000; Young and Davis, 2001; Shore, 2005). The DCN molecular layer consists of excitatory parallel fibers innervating both “cartwheel” interneurons and “fusiform” principal neurons. Cartwheel cells synapse onto themselves (Mugnaini et al., 1980) and strongly inhibit fusiform cells through a disynaptic inhibitory loop (Davis et al., 1996) (Supp. Fig. 1A).

In our studies, we show that the cellular, molecular and functional architecture of EC signaling in the DCN is distinct. Unlike in the hippocampus, cerebellum and striatum, we found that EC-mediated synaptic suppression in the auditory brainstem is more prominent in excitatory than inhibitory inputs. Quantitative electron microscopical (EM) studies revealed that this finding can be explained by lower density of CB1Rs in glycinergic terminals compared to CB1R density at glutamatergic terminals. EM studies also revealed that DGLα and DGLβ, the synthesizing enzymes that produce ECs in the DCN, are preferentially located in the spines of cartwheel cells. One functional
consequence of the cellular and molecular architecture of EC signaling is the modulation of feedforward inhibition. EC signaling in the DCN leads to decreased feedforward inhibition and increased ratio of excitation over inhibition in fusiform cells. Therefore, modulation of circuit properties arises from cell-specific and input-specific EC signaling.

Materials and Methods

Electrophysiology. Coronal brain slices were made from ICR mice (P18-P26). The preparation and use of coronal slices containing DCN has been described in detail (Tzounopoulos et al., 2004). Animals were sacrificed according to methods approved by the Institutional Animal Care and Use Committee of Rosalind Franklin University and the University of Pittsburgh (part of the work was completed at Rosalind Franklin University). Single cells were visualized with IR interference contrast optics and recorded using patch pipettes in either voltage- or current-clamp modes. Cells in the DCN’s molecular and fusiform cell layers were identified on the basis of morphological and electrophysiological criteria (Supp. Fig. 1, Zhang and Oertel, 1993; Manis et al. 1994; Tzounopoulos et al., 2004). The external solution contained (in mM): 130 NaCl, 3 KCl, 1.2 KH2PO4, 2.4 CaCl2, 1.3 MgSO4, 20 NaHCO3, 3 HEPES, and 10 glucose; saturated with 95% O2/5% CO2. For voltage clamp experiments pipettes were filled with a Cs+-based solution containing (in mM): 130 CsGluconate, 10 CsCl, 2 MgCl2, 0.16 CaCl2, 0.5 EGTA, 10 HEPES, 4 Na2ATP, 0.4 NaGTP, and 14 Tris-Creatine phosphate. For current clamp experiments, pipettes were filled with a K+-based internal solution containing (in mM): 113 K-gluconate, 4.5 MgCl2, 14 trisphosphocreatine, 9 HEPES, 0.1 EGTA, 4 NaATP, 0.3 tris-GTP, 10 sucrose. Glycinergic IPSC/Ps were recorded with a pipette
solution that had an elevated [Cl\(^-\)] of 16.7 mM with parallel reduction in gluconate. All the internal solutions were adjusted to pH 7.3, 300 mOsmol. Whole-cell recordings were performed at 31-33°C. For voltage-clamp experiments, series resistance was monitored throughout the experiment from the size and shape of the capacitive transient in response to a 5 mV hyperpolarization. Input resistance was calculated from the sustained response to the same step. Experiments were not included if the series and/or input resistance changed more than 20% throughout recording. Excitatory or inhibitory postsynaptic responses were evoked by stimulating parallel fiber tracts with voltage pulses (100μs, 7-30 V). During DSE or DSI induction, the baseline EPSCs or IPSCs were acquired at a stimulation frequency of 0.67Hz (every 1.5s), followed by a depolarization of either 1s or 5s to 10mV delivered to the postsynaptic cell. Subsequent EPSCs or IPSCs were continuously collected at 0.67Hz. The amplitude of EPSCs or IPSCs was measured and averaged from 5-6 sweeps in each cell, then normalized to the average value before depolarization. DSE or DSI was reported as a percentage of average EPSC or IPSC 1-3s after depolarization versus before depolarization, unless stated otherwise. Pairing of PSPs with postsynaptic action potential was used to induce pairing-evoked suppression of excitation or inhibition (PSE or PSI). This protocol involved five pairs of EPSP or IPSP, coupled with a current-evoked spike 5 ms later, delivered at 100 ms intervals followed by a 5 s pause, and repeated five times (Fig. 2A). EPSPs or IPSPs were collected every 1.5s before and after pairings. The slope of EPSPs or IPSPs was measured and averaged from 5-6 sweeps, then normalized to baseline. Control experiments were interleaved between experiments testing the effect of different pharmacological applications. To study excitatory synaptic responses, 20 μM SR95531 and 0.5 μM strychnine were bath applied
to block GABAergic and glycinergic transmission. To study glycinergic transmission, 20 μM SR95531 and 20 μM NBQX were bath applied to block GABAergic and glutamatergic inputs respectively. SR95531, NBQX and strychnine were dissolved in water. AM-251, RHC-80267 (RHC), tetrahydrolipstatin (THL) and WIN-55,212-2 (WIN) were dissolved in DMSO. The final concentration of DMSO was kept less than 0.1%. SR95531, NBQX and AM-251 were purchased from Ascent Scientific. Strychnine, RHC and THL were purchased from Sigma-Aldrich. WIN was purchased from Tocris Cookson. Data were acquired and analyzed using pClamp10.1. All means are reported ± SEM. Statistical comparisons were made using two-tailed Student’s t tests. Statistical significance was based on p values < 0.05.

**Electron Microscopy.** The handling of the animals prior to and during the experimental procedures was approved and supervised by the University of Connecticut IACUC and followed NIH guidelines. For structural analysis, 4 mice (P22) were used. Mice were anesthetized with a mixture of ketamine 60 mg/kg and xylazine 6.5 mg/kg. After checking anesthetic depth, mice were perfused with 4% paraformaldehyde and 0.5% gluraldehyde in 0.12 M phosphate buffer (pH 7.2) for 10 min. Low glutaraldehyde fixation was followed by either preembedding immunocytochemistry or freeze-substitution as previously described (Rubio and Wenthold, 1997; Tzounopoulos et al., 2007). The identification at the electron microscope of cartwheel and fusiform cells and inhibitory synaptic endings on those neuronal types was done as previously described (Rubio and Juiz, 2004; Rubio, 2006; Tzounopoulos et al., 2007)

**Preembedding immunohistochemistry and freeze-substitution and postembedding immunogold labeling.** Two mice were used for preembedding immunocytochemistry of
DGL\(\alpha\) and DGL\(\beta\) in the DCN. Preembedding immunocytochemistry for the two enzymes was performed as previously described (Rubio and Wenthold, 1997; Rubio et al., 2008). Both antibodies were rabbit polyclonal and were a gift from Dr. Ken Mackie. For the detection of DGL\(\alpha\), DGL\(\beta\), CB1Rs and GlyR\(\alpha1\) receptor subunits, with immunogold labeling after freeze-substitution, a protocol similar to that described in detail elsewhere was used (Rubio and Wenthold, 1997; 1999; Rubio, 2006). Sections at the level of the cochlear nucleus of 2 mice were dissected and processed for freeze-substitution and low-temperature embedding. For postembedding immunocytochemistry, ultrathin sections (80 nm in thickness) on nickel grids were incubated in sodium borohydride and glycine in Tris-buffered saline solution with Triton X-100. After being pre-blocked with serum, the sections were incubated with the affinity purified primary polyclonal antibody for DGL\(\alpha\) and DGL\(\beta\). Primary antibodies were detected with a secondary antibody conjugated to 5nm gold particles in diameter (1:20; Amersham GE Healthcare, Buckinghamshire, UK). No gold particles were observed on mitochondria and myelin sheets. Double postembedding immunogold labeling was performed to determine the subcellular localization of CB1R and GlyR\(\alpha1\) on cartwheel cell inhibitory synapses in the mouse DCN using a polyclonal antibody for CB1R (gift from Dr. Ken Mackie; Tzounopoulos et al., 2007) and a mouse monoclonal antibody for GlyR\(\alpha1\). Two different sizes of gold particles were used to localize each protein. Control sections were prepared either in the absence of the primary antibody during the incubation step. No gold particles were observed on the ultrathin sections after the control procedure (data not shown). Preadsorption for DGL\(\alpha\), DGL\(\beta\) and CB1R was done as previously described (Tzounopoulos et al., 2007). Ultrathin sections were analyzed with a TECNAI G2 Spirit
Biotwin TEM. The images were captured with an AMT CCD camera at 49,000x, 68,000x or 98,000x magnification. Image processing was performed with Adobe Photoshop using only the brightness and contrast commands to enhance gold particles.

**Quantitative analysis.** The distribution and relative density of the CB1 receptor subunit immunolabeling in the inhibitory synapses on cartwheel cell body and apical dendrites of fusiform cells were determined for 40 synapses as previously described (Tzounopoulos et al., 2007). For the quantitative analysis of CB enzymes on cell bodies, apical dendrites and spines of cartwheel and fusiform cells, a total of 200 (DGL\(\alpha\)) and 340 (DGL\(\beta\)) gold particles were analyzed. Sample included per cell type and antibody, 30 spines, 8 dendrites and 3 cell bodies. The data contained intracellular and plasma membrane labeling. NIHScion Imaging software was used to trace the corresponding plasma membranes and areas. Two-tailed tests (assuming unequal variance) were used for statistical comparison.

**Results**

**Differential Strength of EC-Mediated Modulation of Excitatory Inputs and Lack of EC-Mediated Modulation of Inhibitory Inputs in Cartwheel and Fusiform Cells**

DSE and DSI represent a transient decrease in synaptic strength of excitatory and inhibitory inputs that is mediated by endocannabinoids (Wilson and Nicoll, 2001; Kreitzer and Regehr, 2001b; Ohno-Shosaku et al., 2001). DSE and DSI were used to reveal the relative strength of endocannabinoid signaling in excitatory and inhibitory terminals innervating cartwheel and fusiform cells. Application of 1s depolarization to 10
mV in cartwheel cells led to a decrease in the synaptic strength of parallel-fiber evoked EPSCs that lasted ~10-15 seconds (Fig. 1A). Application of 1s depolarization to 10 mV to fusiform cells revealed DSE that was much smaller than in cartwheel cells (Fig. 1B, E). However, increasing the depolarization to 5s revealed DSE in fusiform cells, while 5s depolarization did not induce any further DSE in cartwheel cells (Fig. 1A, B, E). DSE in cartwheel and fusiform cells was mediated by ECs, as it was blocked by the selective CB1R antagonist AM-251 (1μM, data not shown, see also Tzounopoulos et al., 2007). The differential dependence of the DSE magnitude on the depolarizing pulse reflects, at least partially, higher levels of CB1R expression in presynaptic terminals innervating cartwheel compared to fusiform cells (Tzounopoulos et al., 2007). This cell-specific modulation may reflect cell-specific ability to release ECs and/or differential postsynaptic localization of EC synthesizing machinery in relationship to the presynaptic site of EC action.

To understand the physiological role of EC signaling in regulating the output of the DCN, we examined EC signaling in inhibitory synapses in the molecular layer of the DCN. Fusiform cells receive glycinergic inputs from cartwheel cells, and cartwheel cells send local axons to neighboring cartwheel cells. One second and/or 5s depolarization in cartwheel and fusiform cells failed to induce DSI to glycinergic inputs (Fig. 1C, D, F). The first IPSC in all the graphs including IPSCs, as well as the first IPSC after 5s depolarization, was bigger than subsequent responses as a result of frequency-dependent depression of glycinergic IPSCs (Supp. Fig. 2). To determine whether DSI is occluded by the frequency-dependent (presumably presynaptic) depression of IPSCs, we performed DSI experiments at lower frequencies (0.2 Hz) (Supp. Fig. 3A, B) and at spontaneous
IPSCs (Supp. Fig 3C, D). DSI was not observed in either case, indicating that occlusion can not explain the lack of DSI. The absence of DSI at glycinergic synapses is surprising, as in other brain areas, including the hippocampus, the cerebellum and the striatum, inhibitory synapses are more sensitive to endocannabinoid signaling than excitatory synapses as a result of higher levels of CB1Rs at inhibitory boutons (Ohno-Shosaku et al., 2002; Brenowitz et al., 2006; Yoshida et al., 2006; Uchigashima et al., 2007).

While DSE and DSI represent a widely used assay for determining the strength of EC signaling at different cell types and at different synaptic inputs, the physiological relevance of 1-3 seconds depolarization may not be as clear. Therefore, to determine whether our conclusions about cell- and input-specific EC signaling are physiologically relevant, we also used more physiological, current clamp protocols. To elicit EC release, we took advantage of our previous findings showing cell-specific induction of EC signaling by pairing PSPs with postsynaptic APs (Tzounopoulos et al., 2004, 2007). These studies revealed opposing forms of spike timing dependent plasticity (STDP) of parallel fiber inputs to cartwheel and fusiform cells (Tzounopoulos et al., 2004, 2007). In fusiform cells, APs evoked 5 ms after parallel-fiber EPSPs led to long-term potentiation (LTP) that was mediated by calcium calmodulin-dependent protein kinase II-mediated (CaMKII). However, the same EPSP-spike protocol led to EC-mediated long-term depression (LTD) in cartwheel cells, suggesting cell-specific engagement of EC signaling (Tzounopoulos et al., 2007). To trigger short-term, pairing-evoked suppression of excitation (PSE), we used a milder pairing protocol. By reducing the number of EPSP-AP pairings (same as previously used protocol, but the set was repeated 4 instead of 9 times,
Fig. 2A), we were able to induce PSE in cartwheel cells (Fig. 2C). PSE resembled DSE in magnitude and kinetics. In addition, PSE was blocked by AM-251, indicating that it is mediated by EC signaling (Fig. 2C). However, the same pairing protocol did not induce PSE in fusiform cells (Fig. 2D). Moreover, in agreement with our DSE/I studies, the pairing protocol had no effect on the inhibitory inputs on cartwheel and fusiform cells (Fig. 2E, F). Taken together, these results indicate that the DCN displays cell-specific EC signaling: excitatory inputs to cartwheel cells are more sensitive to EC signaling than excitatory inputs to fusiform cells. In addition, our studies indicate that the DCN also displays input-specific EC signaling as the inhibitory inputs to both cell types are not sensitive to EC release. While we know that both fusiform and cartwheel cells release endocannabinoids that mediate DSE (Fig. 1A, B), we were puzzled by our inability to observe DSI. However, the absence of DSI could be explained by the absence or the reduced expression of CB1Rs at glycinergic terminals.

**Lack of EC Signaling in Inhibitory Terminals is Due to Low Levels of Expression of CB1Rs in Glycinergic Inhibitory Terminals**

To determine whether glycinergic terminals express CB1Rs, we used electrophysiological and histological approaches. First, we investigated the sensitivity of glycinergic synapses on cartwheel and fusiform cells to an agonist of CB1R. When WIN-55, 212-2 (WIN, 2 μM) was bath applied, a quantitatively similar decrease of synaptic strength of glycinergic transmission was observed in both cartwheel and fusiform cells (Fig. 3A). This decrease was quantitatively similar to the decrease observed in the excitatory inputs of cartwheel and fusiform cells (Tzounopoulos et al.,
However, when the concentration of WIN was reduced to 50 nM (Fig. 3B), no effect was observed in the glycinergic IPSCs of cartwheel and fusiform cells. Our previous studies have revealed that bath application of 50nM WIN led to a decrease in the strength of excitatory inputs to cartwheel cells (data not shown, Tzounopoulos et al., 2007). One way to interpret these findings is that glycinergic terminals express CB1Rs but to a lesser extent than parallel fiber terminals to cartwheel cells. While a full dose-response curve for WIN on isolated inhibitory and excitatory inputs would provide further confirmation for our hypothesis, previous studies have demonstrated that it is impractical to perform a full dose-response curve because it is difficult to control the concentrations of lipophilic agonists such as WIN within a brain slice (Brown et al., 2004). The highly lipophilic WIN compound equilibrates slowly in slice tissue, and access to different synaptic sites may be affected by multiple factors such as the depth of recordings and the relative amount of glial material at a given synapse. While we believe that our observed differential sensitivity to 50nM WIN between excitatory and inhibitory synapses is not due to differential penetration of the drug, as we were careful not to record from fusiform and cartwheel cells located in deeper layers of the slice, we also used quantitative EM studies to determine whether glycinergic terminals express lower amounts of CB1 receptors.

Double postembedding immunogold localization was performed to determine the distribution and the abundance of CB1Rs in inhibitory synapses in the molecular layer. Five-nm gold particles labeling the GlyRα1 subunit were apparent within glycinergic terminals onto both cell types (Fig 3C). Ten-nanometer gold particles labeling CB1Rs
appeared evenly distributed in the axoplasm and along the presynaptic plasma membrane of these glycinergic endings. The distribution of presynaptic gold particles was quantified as shown in the cartoon in Figure 3D; gold particles were classified as being associated with membrane facing the postsynaptic density (PSD) at the periphery of the PSD, or in extrasynaptic membrane areas. Particles had to be within 40 nm of presynaptic plasma membrane to qualify as membrane labeling (see Materials and Methods). This analysis revealed that CB1R particle densities obtained were very low at glycinergic terminals (values ranged from 0.2 per μm ± 0.1 SEM at non-synaptic plasma membranes to 0.5 per μm ± 0.2 SEM at the plasma membrane facing the postsynaptic membrane). Comparison of these density values with the values obtained for the density of membrane-associated gold particles for CB1R on parallel fiber endings synapsing onto cartwheel cells (dashed lines, Fig. 3D) emphasizes the low levels of CB1R expression in glycinergic terminals. The density of intracellular gold particles was also significantly lower in glycinergic terminals than in parallel fiber excitatory terminals (Fig. 3E). Therefore, these data confirm that differential EC signaling between excitatory and inhibitory terminals is caused by input-selective expression of CB1Rs. In addition, these data confirm the predicted distinct anatomy of EC signaling in the DCN and are consistent with the absence of DSI/PSI under conditions that reveal DSE/PSE.

2-AG Mediates EC Signaling in the DCN

An alternative factor that could contribute to the lack of DSI predicts that the distance between postsynaptic EC production site and presynaptic CB1Rs is different for excitatory and inhibitory inputs in the DCN. According to this hypothesis, the lack of DSI
may result from the longer distance that ECs have to cover in order to modulate potentially distant inhibitory inputs. Inhibitory inputs synapse on the soma and dendritic shafts of fusiform and cartwheel cells, while excitatory inputs synapse onto spines of both cell types (Kane, 1974; Wouterlood and Mugnaini 1984; Smith and Rhode, 1985; Rubio and Juiz, 2004). To determine the release site of ECs we used physiological and histological techniques.

Initially, we determined the synthesizing enzymes that produce ECs in the DCN. The two best characterized ECs are anandamide (AEA) (Devane et al., 1992; Felder et al., 1993; Di Marzo et al., 1994) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Di Marzo et al., 1994). In the hippocampus (Makara et al., 2005; Chevaleyre et al., 2006; Katona et al., 2006; Hashimotodani et al., 1997b), cerebellum (Safo and Regehr, 2005; Szabo et al., 2006; Yoshida et al., 2006), striatum (Uchigashima et al., 2007), and prefrontal cortex (Melis et al., 2004; Lafourcade et al., 2007), 2-AG is the most common EC mediating short- and long-term plasticity. However, AEA is involved in EC-dependent LTD in amygdala and the levels of AEA determine the sign of synaptic plasticity in the striatum (Azad et al., 2004; Ade and Lovinger 2007). Given the similarities of the DCN with the cerebellum (Oertel and Young, 2004), we focused initially on 2-AG. 2-AG is produced by cleavage of diacylglycerol (DAG) by DAG lipase (DGL). DGL exists in two isoforms, α and β, and acts in an activity-dependent manner (Stella et al., 1997; Bisogno et al., 2003; Piomelli, 2003). To determine whether EC signaling in the DCN is mediated by 2-AG synthesis and release, we examined the effect of tetrahydrolipstatin (THL, a DGL inhibitor) on DSE. Bath application of 10 μM THL blocked DSE in cartwheel cells (Fig. 4A, D). Similarly, bath application of another DGL
inhibitor, RHC-80267 (25 µM) (Bisogno et al., 2003; Melis et al., 2004), greatly reduced DSE in cartwheel cells (Fig. 4B, D). DSE induced in fusiform cells with 5s depolarization was also blocked by THL (Fig. 4C, D). These results show that postsynaptic depolarization induces DGL-mediated synthesis of 2-AG, which results in DSE in the parallel fiber inputs of cartwheel and fusiform cells.

**DGLα and DGLβ are Differentially Distributed at Parallel Fiber Synapses on Cartwheel and Fusiform Cells**

To determine the precise 2-AG release site, we examined the localization of its synthesizing enzymes, DGLα and DGLβ. Two different approaches were used: preembedding immunohistochemistry followed by conventional electron microscopy and postembedding immunogold labelling using secondary antibodies conjugated to 5 nm gold particles. Analysis showed that DGLα and DGLβ were differentially distributed in fusiform and cartwheel cells. The 3,3’-diaminobenzidine end product of the immunoperoxidase staining procedure indicates that DGLα is found in cell bodies, dendrites and dendritic spines of cartwheel cells, and in the cell bodies and dendrites of fusiform cells, but not in the spines (Fig. 5A, B). Immunostaining for DGLβ was also observed in the cell bodies and dendrites of fusiform and cartwheel cells, but it was only expressed in the spines of cartwheel cells (Fig. 5E, F). In cartwheel cell spines, immunoreaction for both enzymes was associated with intracellular membranes of endoplasmic reticulum and was restricted to the spine head (Fig. 5A, E). Postembedding immunogold labeling revealed similar distributions (Fig. 5C, D and G, H). To quantify the distribution of DGLα and DGLβ in the different cell compartments, we calculated the
density of gold particles per area of cell bodies, dendrites and spines (Fig. 5I, J). We also quantified separately the cell surface and intracellular DGL immunogold labeling (DGLα: Intracellular: $4.7 \pm 1.6$ gold particles/$\mu m^2$, PSD: $0.4 \pm 0.3$ gold particles/$\mu m$, Plasma membrane: $0.2 \pm 0.1$ gold particles/ micron length; DGLβ: Intracellular: $11.5 \pm 3.2$ gold particles/$\mu m^2$, PSD: $5.4 \pm 1.1$ gold particles/$\mu m$, Plasma membrane: $0.5 \pm 0.2$ gold particles/ micron length).

All together, our data show that DGLα and DGLβ are distributed evenly in the cell body and dendrites of fusiform cells, with no gold labelling in the spines. On cartwheel cells, both enzymes appeared in spines, cell bodies and dendrites, but DGLβ expression in spines was significantly higher than in the other two compartments (Fig. 5J). The distribution of DGLβ enzyme in spines predicts a spatial disadvantage for inhibitory terminals on cartwheel cells, which are located on dendrites or cell bodies, and thus far from the 2-AG production sites. In addition, given that excitatory inputs synapse onto the spines of fusiform cells, the lack of DGL expression in these spines may also explain the weaker DSE observed in fusiform cells.

**Functional Consequences of Cell- and Input-specific EC Signaling on Circuit Properties**

The timing of feedforward, disynaptic inhibition and the ratio of excitation over inhibition determine the duration of the temporal window over which integration of excitatory inputs may occur (Pouille and Scanziani, 2001; Gabernet et al., 2005; Lamsa et al., 2005; Mittmann et al., 2005). To determine the effect of EC signaling on the modulation of the ratio of excitation over inhibition, we recorded EPSCs and glycineergic
disynaptic IPSCs from fusiform and cartwheel cells (the molecular layer of the DCN contains two different disynaptic inhibitory networks, Supp. Fig. 1A). Parallel fiber stimulation evoked an EPSC followed by an IPSC for both cell types (Fig. 6A, B). The AMPA glutamate receptor antagonist blocked the EPSC and the IPSC (Fig. 6A, B), confirming that the inhibitory component represents disynaptic inhibition and not direct stimulation of interneurons. To determine the effect of EC signaling in shaping the balance of excitation and inhibition, we bath applied a low concentration of CB1R agonist (50 nM WIN). Experiments presented here (Fig. 3A, B) and in previous studies (Tzounopoulos et al., 2007) have revealed that application of 50 nM WIN mimics the cell- and input-specific effects of EC signaling in the DCN. Bath application of WIN led to reduction in disynaptic inhibition in fusiform cells (Fig. 6C). This reduction was expected and was due to the cell-specific effect of EC signaling on parallel fiber inputs innervating cartwheel cells (Tzounopoulos et al., 2007). The reduction of the disynaptic IPSC was not associated with a reduction of the EPSC in the EPSC-IPSC sequence (Fig. 6C) and therefore, EC signaling increased the ratio of excitation over inhibition in fusiform cells (Fig. 6E). In cartwheel cells, WIN led to a reduction of disynaptic inhibition that was paralleled by an equal reduction of monosynaptic excitation (Fig. 6D). Therefore, EC signaling, unlike its effects on fusiform cells, maintains the ratio of excitation and inhibition in cartwheel cells (Fig. 6E). Taken together, our results suggest that EC signaling in the DCN contributes to network-specific, activity-dependent modulation of the balance of excitation over inhibition. Such modulation may have important effects on spike timing precision and spike output.
Discussion

In this study, we investigated the subcellular organization and the effects of EC signaling in the DCN synaptic circuitry. EC signaling favors retrograde inhibition of excitatory inputs in the DCN, and thus differs from the organization and functional properties of EC system observed at the cerebellum, hippocampus and striatum. Excitatory and inhibitory inputs found in the molecular layer express CB1Rs; yet, glycinegic inputs express fewer CB1Rs. Localization of EC release sites revealed that in cartwheel cells, inhibitory inputs are distant to EC release sites. Cell- and input-specific EC signaling increases the ratio of excitation over inhibition in principal cells, while it maintains this ratio unchanged in the interneuronal network.

Distinct Anatomical Organization of EC System in the DCN

Anatomical and functional studies have elucidated the subcellular organization of the endocannabinoid signaling molecules in the hippocampus, cerebellum, striatum and prefrontal cortex (Melis et al., 2004; Makara et al., 2005; Safo and Regehr, 2005; Chevaleyre et al., 2006; Katona et al., 2006; Szabo et al., 2006; Yoshida et al., 2006; Hashimotodani et al., 2007a; Lafourcade et al., 2007; Uchigashima et al., 2007). In general, the molecules involved in EC synthesis are co-localized postsynaptically, whereas CB1Rs are located presynaptically (Katona and Freund, 2008). The localization of these elements seems to be region-specific, thus serving the specific computational needs of each area.
In all other systems investigated so far, including the hippocampus, cerebellum and striatum, inhibitory synapses are more sensitive to ECs than excitatory synapses, as a result of higher levels of CB1Rs at inhibitory inputs (Ohno-Shosaku et al., 2002; Brenowitz et al., 2006; Yoshida et al., 2006; Uchigashima et al., 2007). In the hippocampus, excitatory terminals synapse on spines expressing DGLα, while inhibitory terminals synapse onto somatodendritic contacts lacking DGLα (Ohno-Shosaku et al., 2002). However, this spatial disadvantage of inhibitory terminals is compensated by increased sensitivity of inhibitory terminals to 2-AG due to higher levels of CB1R expression. The arrangement and abundance of EC signaling molecules in the DCN differs from other brain regions, thus suggesting distinct functions of EC signaling in the auditory brainstem (Fig. 7). In the DCN, we find that inhibitory glycinergic terminals express lower levels of CB1Rs (Fig. 3). This unconventional expression pattern of CB1Rs leads to a decreased sensitivity of inhibitory terminals to EC signaling under normal activity. Previous studies have reported GABAergic inhibitory terminals completely lacking CB1Rs (Bodor et al., 2005; Fortin and Levine, 2007). In the auditory system, EC-mediated retrograde suppression of excitatory inputs was first reported at the calyx of Held synapse in the medial nucleus of the trapezoid body (MNTB), an auditory brainstem nucleus. Our previous studies revealed the role of EC signaling in determining cell-specific synaptic plasticity in the DCN (Tzounopoulos et al., 2007), while this study addresses the overall strength, organization and synaptic function of EC system in the DCN. This is the first study of EC signaling in glycinergic inhibitory inputs in auditory circuits, although a previous study had reported EC signaling in glycinergic inputs in hypoglossal nucleus synapses (Mukhtarov et al., 2005).
Our physiological and anatomical studies indicate that 2-AG mediates EC signaling in fusiform and cartwheel cells (Figs. 4 and 5). We used specific antibodies for 2-AG-synthesizing enzymes: DGL\(\alpha\) and DGL\(\beta\). DGL\(\alpha\) and DGL\(\beta\) are found on somata and dendrites of cartwheel and fusiform cells (Fig. 5). DGL\(\alpha\) and DGL\(\beta\) are also found on the spines of cartwheel cells, but are absent from the spines of fusiform cells. Recent studies suggest that 2-AG is synthesized by perisynaptic DGL-\(\alpha\) enzymes located on the postsynaptic neuron and then activates presynaptic CB1 receptors (reviewed by Katona and Freund, 2008). Moreover, in the DCN, DGL\(\beta\) is highly concentrated in the spines of cartwheel cells (Fig. 5J). It is not known whether both enzymes have different roles in EC signaling, but given the predominant localization of DGL\(\beta\) in cartwheel cell spines, we hypothesize that this enzyme may be important for the cell-specific, EC-mediated, anti-Hebbian LTD observed in cartwheel cells. Additionally, the lack of DSI in cartwheel cells is due to the low abundance of CB1Rs in glycinergic terminals and to the increased distance of postsynaptic DGL\(\beta\) and presynaptic CB1Rs at glycinergic terminals (Fig. 7).

**Network Consequences of Input- and Cell-Specific EC signaling in the DCN**

Previous *in vivo* studies (Young et al., 1995; Shore, 2005) suggest that DCN cartwheel cells fire rapidly in response to parallel fiber inputs and provide fast and robust feedforward inhibition to fusiform cells. Here we show that 1s depolarization or pairing of EPSPs and APs induce DSE or PSE, respectively, in cartwheel cells, but fail to reveal DSE and PSE in fusiform cells (Figs. 1 and 2). This preferential EC retrograde inhibition at parallel fiber inputs innervating cartwheel cells shifts the effect of parallel fiber
activation in the fusiform cell from IPSP-dominant (feedforward inhibition overwhelms the EPSP) to EPSP-dominant (Fig. 6C, E). The net effect of cell-specific EC signaling would be to bias the system towards the excitation of the output neuron. In the cerebellum, parallel fiber synapses onto stellate cells (feedforward interneurons) share similar responses to EC signaling with parallel fiber synapses to Purkinje cells (PCs), indicating that PF-mediated excitation and stellate cell-mediated feedforward inhibition onto PCs are tightly coordinated (Beierlein and Regehr, 2006; 2007). However, the lack of coordination in the DCN implies different function of EC signaling on auditory circuits. Feedforward inhibition determines the duration of the temporal window over which integration of excitatory inputs may occur (Pouille and Scanziani, 2001; Gabernet et al., 2005; Lamsa et al., 2005; Mittmann et al., 2005). The EC-mediated reduction in feedforward inhibition may allow fusiform cells to effectively integrate incoming excitatory inputs over a longer timing window, thus modulating spike timing precision and promoting certain forms of short- or long-term plasticity. Such novel modulation, by introducing plasticity in spike timing precision, may provide a mechanism for adaptive encoding of auditory inputs.

**EC signaling, Synaptic Plasticity and Tinnitus**

Our previous studies have shown that selective engagement of EC signaling in cartwheel cells and its interaction with the CaMKII signaling cascade mediates cell-specific synaptic plasticity in the DCN (Tzounopoulos et al., 2007). In general, expression of neural plasticity can lead to compensation for loss of function and adaptation to changing
demands. However, plasticity-induced changes can also cause signs and symptoms of disease.

Tinnitus - commonly referred to as ringing in the ears or head - is the perception of sound in the absence of an environmental acoustic stimulus; tinnitus can be a very severe condition as millions of patients are disabled by the persistence and the intensity of this “phantom” sound. Despite the prevalence of tinnitus, the pathophysiology of the disorder is poorly understood. In light of several studies suggesting increased spontaneous activity in DCN cells in animal models of tinnitus (Brozoski et al., 2002; Kaltenbach et al., 1998; Kaltenbach et al., 2000; Kaltenbach et al., 2004; Kaltenbach and Zhang, 2005), a novel hypothesis proposes that activity-dependent mechanisms that change the balance of excitation and inhibition on fusiform cells could lead to hyperactivity of fusiform cells, via plasticity-like mechanisms (Tzounopoulos, 2008). Consistent with this hypothesis, recent studies showed that CB1Rs are down-regulated in the cochlear nucleus in animal model of tinnitus (Zheng et al., 2007). Therefore, determining the functional role of EC signaling in determining the output of the DCN may also lead to a better understanding on the cellular mechanisms underlying tinnitus.
**Figure Legends**

**Figure 1. Cell- and input-specific EC signaling: differential DSE in cartwheel and fusiform cells and lack of DSI in both Cell Types.**

**A,** Time course of DSE, induced by 1s or 5s depolarization in cartwheel cells (CWC). Traces represent average EPSC 1-3 sec before and 1-3 sec after depolarization.

**B,** Time course of DSE, induced by 1s or 5s depolarization in fusiform cells (FC). Traces represent average EPSC 1-3 sec before and 1-3 sec after depolarization.

**C,** Time course of DSI, induced by 1s or 5s depolarization in cartwheel cells (CWC). Traces represent average IPSC 1-3 sec before and 1-3 sec after 1s depolarization, or 2-3 sec after 5s depolarization.

**D,** Time course of DSI, induced by 1s or 5s depolarization in fusiform cells (FC) Traces represent average IPSC 1-3 sec before and 1-3 sec after 1s depolarization, or 2-3 sec after 5s depolarization.

**E,** Summary graph showing comparison of average DSE in cartwheel and fusiform cells. Average DSE was calculated as average EPSC 1-3s after/ before depolarization (cartwheel cells: DSE after 1s depolarization: 44% ± 5%, n = 5; DSE after 5s depolarization: 58% ± 7%, n = 5; fusiform cells: 1s, 18% ± 2%, n = 6; 5s, 41% ± 6%, n = 5, p<0.05).

**F,** Summary graph showing comparison of average DSI in cartwheel and fusiform cells. Average DSI was calculated as average IPSC 1-3s after/ before depolarization (cartwheel cells: DSI after 1s depolarization: 4% ± 3%, n = 8; DSI after 5s depolarization: 8% ± 1%, n = 5; fusiform cells: 1s, -2% ± 6%, n = 7; 5s, -14% ± 4%, n = 5).

All means are reported ± SEM.
Figure 2. Pairing of PSPs and spikes reveals similar cell- and input-specific EC signaling in the DCN.

A, EC signaling was induced by a protocol comprising of five pairs of subthreshold EPSPs and current evoked spikes delivered 5 ms later. These five pairs were delivered at 100 ms intervals, followed by a 5s pause, and repeated a total of 5 times.

B, Example of a cell’s (cartwheel left, fusiform right) responses to pairing of a subthreshold EPSP with a current-evoked spike delivered 5 ms later.

C, Time course of EC-mediated inhibition of parallel fiber inputs to cartwheel cells (CWC), induced by the pairing protocol (control: average EPSP 1-3s after/before pairing: 81% ± 3%, n = 5, p<0.05; AM-251: average EPSP 1-3s after/before paring: 125% ± 8%, n = 5, p < 0.05). Traces represent average EPSP 1-3 sec before and 1-3 sec after pairing.

D, Time course of EC-mediated inhibition of parallel fiber inputs to fusiform cells (FC), induced by the pairing protocol (control: average EPSP 1-3s after/before pairing: 102% ± 4%, n = 6; AM-251: average EPSP 1-3s after/before paring: 107% ± 4%, n = 5). Traces represent average EPSP 1-3 sec before and 1-3 sec after pairing.

E, Time course of EC-mediated inhibition of glycinergic inputs to cartwheel cells (CWC), induced by the pairing protocol, (control: average IPSP 1-3s after/before pairing: 99% ± 3%, n = 5; AM-251: average IPSP 1-3s after/before paring: 98% ± 8%, n = 5). Traces represent average IPSP 1-3 sec before and 1-3 sec after pairing.

F, Time course of EC-mediated inhibition of glycinergic inputs to fusiform cells (FC), induced by the pairing protocol (control: average IPSP 1-3s after/before pairing: 98% ±
4%, n = 5; AM-251: average IPSP 1-3s after/before pairing: 103% ± 12%, n = 5). Traces represent average IPSP 1-3 sec before and 1-3 sec after pairing. All means are reported ± SEM.

**Figure 3. Low expression of CB1Rs in glycinergic inhibitory synaptic endings.**

**A,** Time course of 2 µM WIN-55,212-2 block of transmission in cartwheel (CWC) and fusiform cells (FC). Average WIN block was calculated as average IPSC 25-30 min after /before WIN application: cartwheel cells: 40% ± 2%, n = 4; fusiform cells: 40% ± 3%, n = 4.

**B,** Time course of 50 nM WIN-55,212-2 block of transmission in cartwheel (CWC) and fusiform cells (FC). Average WIN block was calculated as average IPSC 25-30 min after /before WIN application: cartwheel cells: 103% ± 2%, n = 5; fusiform cells: 91% ± 3%, n = 4).

**C,** Electron micrographs show double postembedding immunogold labeling for CB1Rs (10 nm) and GlyRα1 (5 nm) on cartwheel cell axons on cartwheel and fusiform cells. Gold particles for GlyRα1 are only observed at the postsynaptic membranes and intracellularly in the cell bodies. Scale bar: 0.2 μm.

**D,** Histogram showing the distribution and the density of gold particles for CB1Rs at the plasma membrane of cartwheel cell endings. Dashed lines represent the density of membrane-associated gold particles for CB1Rs on parallel fiber endings synapsing onto cartwheel cells as described by Tzounopoulos et al., 2007.
Figure 4. **2-AG mediates endocannabinoid signaling in cartwheel and fusiform cells.**

**A**, Time course of DSE in tetrahydrolipstatin (THL, inhibitor of 2-AG synthesis) in cartwheel cells.

**B**, Time course of DSE in RHC-80267 (RHC, inhibitor of 2-AG synthesis) in cartwheel cells.

**C**, Time course of DSE in THL in fusiform cells.

**D**, Summary graph showing comparison of average DSE in control and DGL blockers, for cartwheel and fusiform cells. Average values were calculated as indicated in Figure 1 (cartwheel cells: control DSE: 67% ± 5.5%, n = 7; DSE in THL: .5% ± 3.5%, n = 6, p < 0.05; DSE in RHC: 33% ± 6%, n = 6, p < 0.05; fusiform cells: control DSE: 29.5% ± 4%, n = 4; DSE in THL: 9% ± 2.5%, n = 7, p < 0.05)

All means are reported ± SEM.

Figure 5. **Differential distribution of DGLα and DGLβ at parallel fiber synapses on fusiform and cartwheel cells.** Electronmicrographs show preembedding immunohistochemistry (A-B and E-F) and postembedding immunogold labeling (C-D and G-H) for DGLα and DGLβ.
**A, B and E, F**, Electron dense reaction for DGLα or DGLβ is observed in spines (s) of cartwheel cells but not in spines of fusiform cells. Dendrites of both cell types contain positive immunoreaction associated to intracellular organelles. The PSD of cartwheel cells receiving parallel fiber (PF) synapses also present electron dense reaction but only for DGLβ. Scale bar: 0.5 μm.

**C, D and G, H**, Post-embedding immunogold labeling for DGLα and DGLβ in spines (s) of cartwheel and fusiform cells. Only spines of cartwheel cells present gold particles for both enzymes.

**G**, Electron micrograph showing the localization of gold particles for DGLβ at the postsynaptic density of cartwheel cell spines receiving PF. Arrows: point out the localization of the enzymes. Scale bar: 0.2 μm.

**I, J**, Histograms showing the density of gold particles for both enzymes, per area of cell bodies, dendrites and spines. *** P < 0.005.

**Figure 6.** Cell- and input-specific EC signaling modulates the balance of excitation/inhibition in the auditory brainstem

**A, B** EPSC-IPSC sequence evoked in fusiform and cartwheel cells by parallel fiber stimulation. AMPA-type receptor antagonist (NBQX, 10 μM) blocks both inward (EPSC) and outward (IPSC) current, confirming the disynaptic origin of the IPSC.

**C, D** Effect of 50nM WIN on EPSC-IPSC sequence in a fusiform and cartwheel cells. 50 nM WIN reduces disynaptic IPSC, but does not reduce monosynaptic EPSC in fusiform cells. 50 nM WIN reduces disynaptic IPSC and monosynaptic EPSC in fusiform cells.
E, Summary graph showing the changes in EPSC/IPSC ratio between control and 20-30 min after WIN application. Ratios were normalized to control values (fusiform cells: 150% ± 10%, n=7, p < 0.05; cartwheel cells: 115% ± 10%, n=5)

**Figure 7. Schematic illustration showing the anatomical organization of EC signaling in the excitatory and inhibitory inputs of the DCN.**

Cartoon illustrating lower levels of expression of CB1Rs in glycinergic terminals compared to glutamatergic terminals. CB1Rs are more abundant in parallel fiber terminals innervating CWCs. Both CWC and FC release 2-AG and express DGLα and DGLβ. However, expression of DGLβ shows a preference for the spines of CWC. The distance that 2-AG has to travel to activate CB1Rs is longer, on average, for the glycinergic terminals. Taken together, the architecture of the EC system in the DCN is different from the ones reported for the hippocampus, cerebellum and striatum and is consistent with lack of DSI under protocols that induce DSE. Ex: Excitatory, glutamatergic terminals; In: Inhibitory, glycinergic terminals; PF: Parallel fibers

**Supplemental Figure 1. DCN circuitry and characteristic responses of fusiform and cartwheel cells.**

A, DCN circuitry: Parallel fibers, the axons of granule cells, contact spines on the dendrites of cartwheel cells (glycinergic, inhibitory interneurons) and apical dendrites of fusiform cells (principal, output neurons). Cartwheel cells terminate locally, contacting fusiform cells and other cartwheel cells.
B, Characteristic responses of cartwheel and fusiform cells to current injection: cartwheel cells fire combinations of simple and complex spikes while fusiform cells fire simple spikes.

C, In cell-attached mode fusiform cells showed simple spiking and cartwheel cells showed complex spiking (this method was used to determine cell identity in experiments performed with Cs⁺-based solutions)

Supplemental Figure 2. Frequency-dependence of IPSCs in cartwheel and fusiform cells.

A, Representative IPSCs recorded at different stimulation frequencies in cartwheel cells (IPSCs represent steady state responses).

B, Summary of IPSC amplitude in response to different stimulating frequencies in cartwheel cells. Average IPSC amplitude normalized to the average IPSC obtained at 0.1Hz (0.05Hz: 113% ± 1%; 0.2Hz: 76% ± 7%; 0.4Hz: 65% ± 6%; 0.67Hz: 61% ± 7%; 1Hz: 51% ± 4%, n=5-8 for all frequencies).

C, Representative IPSCs collected at different stimulation frequencies in fusiform cells

D, Summary of IPSC amplitude in response to different stimulation frequencies in fusiform cells. Average IPSC amplitude normalized to the average IPSC obtained at 0.1Hz (0.05Hz: 111% ± 8%; 0.2Hz: 82% ± 3%; 0.4Hz: 77% ± 6%; 0.67Hz: 64% ± 7%; 1Hz: 61% ± 5%, n= 5-8 for all frequencies).

All means are reported ± SEM.
Supplemental Figure 3. Frequency dependence of IPSCs does not occlude DSI.

A, Time course of DSI, induced by 5s depolarization in cartwheel cells (CWC). IPSCs are evoked every 5s (average IPSC 5-15s after/before 5s depolarization: 99% ± 5%, n = 5).

B, Time course of DSI, induced by 5s depolarization in fusiform cells (FC). IPSCs are evoked every 5s (average IPSC 5-15s after/before 5s depolarization: 99% ± 4%, n = 5).

C, Time course of DSI, induced by 5s depolarization in CWCs. In this case the frequency of action-potential-dependent spontaneous IPSCs (sIPSCs) is plotted (average sIPSC frequency 1-5s after/before 5s depolarization: 99% ± 7%, n = 5)

D, Time course of DSI, induced by 5s depolarization in FCs. In this case the frequency of action-potential-dependent spontaneous IPSCs (sIPSCs) is plotted (average sIPSC frequency 1-5s after/before 5s depolarization: 99% ± 8%, n = 5)
REFERENCES


Schofield BR, Coomes DL (2005a) Projections from auditory cortex contact cells in the cochlear nucleus that project to the inferior colliculus. Hear Res 206:3-11.


Zhao et al., Fig. 2
**A**

WIN 2 μM

IPSC (% control)

Time (min)

**B**

WIN 50 nM

IPSC (% control)

Time (min)

**C**

Cartwheel Cells

Fusiform Cells

Cell body

CB1 (10nm) + GlyRα1 (5nm)

**D**

Density of gold particles/μm

Facing PSD

Periphery PSD

Extrasynaptic PM

**E**

Density of gold particles/μm²

Intracellular PF

Intracellular inhibitory

Zhao et al., Fig. 3
Zhao et al., Fig. 4
Zhao et al., Fig. 6
Zhao et al. Fig. 7